THE EFFECT OF X-IRRADIATION ON NUCLEASE ACTIVITY AND RESPIRATION OF TETRAHYMENA GELEII W[1]

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The marked resistance of unicellular organisms to the action of ionizing radiations, as opposed to the low resistance of mammals, has been known for some time. However, little information is available concerning the response of specific biochemical processes of single cell animals to irradiation. The process which has probably been most extensively investigated is cell respiration. Barron, Gasvoda and Flood (1949a) summarized the early papers in this field and showed that the respiration of dilute suspensions of sea urchin sperm could be inhibited from 10–66 per cent with from 100–20,000 r. The inhibition was believed to be due to the action of stable organic peroxides produced by x-irradiation of sea water (Barron *et al.*, 1949b). They also found that the utilization of succinate and acetate was impaired in x-irradiated sperm. Billen, Stapleton and Hollaender (1952) observed that a dose of 60,000 r, while decreasing the number of viable cells of a suspension of *E. coli* B/r by more than 99 per cent, had no effect on the initial respiratory rate but decreased the oxygen consumption afterwards. The presence of pyruvate or succinate was more effective than glucose in prolonging the initial period of normal activity.

As part of a general program involving the effects of x-irradiation on enzyme systems of various animals, and specifically, in an effort to extend the knowledge of biochemical changes in irradiated unicellular organisms, the enzymes ribonuclease (RNase) and desoxyribonuclease (DNase) and respiration were selected for the present study. Data obtained by Roth, Eichel *et al.* (1953) suggested that the RNase of rat liver was a highly labile enzyme under conditions of whole body irradiation.

Materials and Methods

Cultures. Pure stock cultures of *Tetrahymena geleii* W were maintained at room temperature in 50-ml. Erlenmeyer flasks containing 10–15 ml. of 2 per cent Difco proteose-peptone plus 0.2 per cent Difco yeast extract. Transfers were made each day from a 48-hour culture using a 2 mm. platinum loop. Cultures for experimental use were prepared daily by inoculating one ml. of the 48-hour stock cultures into a two-liter Erlenmeyer flask containing 750 ml. of medium.

Preparation of cells, homogenates and dry weight determinations. For use, the Tetrahymena from 750 ml. of 48-hour cultures were separated according to the method of Seaman (1949) using 50-ml. conical centrifuge tubes. Our cells,

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however, were concentrated in a volume of 25 ml. Homogenates were prepared by diluting an aliquot of the concentrate (usually 5 ml.) with an equal volume of distilled water and then grinding with silica in an all-glass homogenizer at 0–2° C. until microscopic examination revealed no unruptured cells. The homogenates were centrifuged at 5000 r.p.m. in an International No. 1 centrifuge for five minutes to remove the silica and cell fragments. Dry weight determinations were made in triplicate at 80° C. (Seaman, 1951) on the whole cells or homogenates. The dry weights of one ml. of the whole cell concentrates and one ml. of the 1:2 homogenates averaged 9.16 (6.57–13.15) and 3.68 mg. (3.00–4.04), respectively.

Irradiation procedure. Four ml. of the concentrated suspensions or homogenates were added to plastic containers ($7/8 \times 7/8 \times 11/16$ inches) which were then packed in ice in plastic Petri dishes to be irradiated or used as controls. The radiation factors have been described (Wichterman, 1948). The radiation flux was 6300 r per minute. When samples were irradiated at 300,000 or 500,000 r, the ice was renewed every 25 minutes.

Respiration studies. Oxygen consumption by the whole cells was measured at 27° C. using standard Warburg manometry. Each vessel contained one ml. of 0.1 *M* Na_2HPO_4–KH_2PO_4 buffer, pH 7.4, one ml. of cell concentrate and 0.8 ml. of distilled water in the main compartment and 0.2 ml. of 10 per cent NaOH in the center well. The vessels were allowed to equilibrate 10 minutes, the stopcocks closed and readings taken at either 10- or 15-minute intervals for one hour. The vessels were shaken at a rate of 120 oscillations per minute and the gas phase was air. Generally, 20–30 minutes elapsed between the completion of irradiation and the first reading. The average QO_2 value of normal cells obtained by assay of 18 different cultures was 25.9 (16.5–34.1), while the average oxygen consumption per million cells was 109 $mm.^3$ (80–150).

Nuclease determinations. RNase and DNase in homogenates of Tetrahymena were assayed at 25° C. by a modification of the turbidimetric method of McCarty (1948). For the RNase tests, the system consisted of 10 ml. of 0.2 per cent sodium ribonucleate (Nutritional Biochemicals), 10 ml. of veronal-acetate buffer, pH 5.85, and a suitable volume of 1:2 homogenate (usually 2 ml.) added at zero time. In every case, the final volume was made up to 25 ml. with distilled water. The components of the DNase system were 10 ml. of 0.2 per cent sodium desoxyribonucleate containing $MgSO_4$ to give a final Mg^{++} ion concentration of 0.003 *M* in 25 ml., 10 ml. of veronal-acetate buffer, pH 5.20, and 5 ml. of a 1:2 hemogenate. Four-ml. samples of each were withdrawn at various intervals (usually 2, 10, 20, 30, 40 and 60 minutes), added to 4 ml. of 1 *M* HCl in 18×150 mm. test tubes and the turbidity read after one minute in a Lumetron photoelectric colorimeter (Model 401) at 420 mμ against a distilled water blank. Under the conditions of the test, the RNase reaction rate was generally linear for 40–60 minutes while the DNase exhibited linearity during the entire 60 minutes. For each enzyme the pH employed is based on preliminary observations only, and does not unequivocally represent the pH of optimum activity. One unit of enzyme activity is defined as that amount of enzyme which, in 25 ml. of test solution, causes a decrease in optical density of 0.1 in 60 minutes. The specific activity is equal to the number of units per mg. dry weight.

Experimental Results

Respiration studies. The QO_2 values of normal and irradiated cells exposed to 300,000 and 500,000 r are summarized in Table I. It can be seen that respiration was usually significantly inhibited at 300,000 r, the average of twelve runs being approximately 30 per cent. No explanation can be given for the wide range of inhibition observed. In two cases, the inhibition was less than 10 per cent. In two instances assays were performed on irradiated cells after 24 hours. In one experiment, a 25 per cent increase in respiration of the irradiated cells was observed, and in the second, the increase amounted to 125 per cent. When the radiation level was raised to 500,000 r, the average inhibition was 40 per cent.

Table I

The effect of x-irradiation on the respiration of Tetrahymena at 27° C.

Dose (r)	Time after irradiation (hrs.)	Control QO_2	Irradiated QO_2	Change (per cent)
300,000	0	22.2	20.3	− 9
....	0	23.7	21.9	− 8
....	0	16.5	13.6	−18
....	0	34.0	25.5	−25
....	0	32.9	27.9	−15
....	0	23.8	16.7	−30
....	0	34.1*	22.7*	−33
....	0	27.8	17.6	−36
....	0	24.3	13.1	−46
....	0	30.1	15.7	−48
....	0	30.0	22.4	−25
	24	10.0	14.2	+25
....	0	24.4	12.8	−48
	24	8.0	18.0	+125
500,000	0	23.3	12.4	−47
	2.5	25.4	6.2	−76
....	0			−29**
....	0	28.8	16.0	−44
	8	10.8	2.6	−76

* Data obtained with *T. geleii* strain E.
** Based on mm.^{3} oxygen consumed.

In two instances when assays were run at 2.5 and 8 hours after exposure, the inhibition rose to 76 per cent.

It is well known that several substances will diminish radiosensitivity by interaction with active radicals produced by ionizing radiations (Patt *et al.*, 1950; Smith *et al.*, 1950; Chapman and Cronkite, 1950; Hollaender, Stapleton and Martin, 1951; Forssberg, 1950). Some of these compounds were tested for their ability to protect Tetrahymena exposed to 300,000 r. For each experiment, 2 ml. of the cell concentrates were diluted with 2 ml. of each of the neutralized test compounds, or with 2 ml. of distilled water, and then immediately irradiated. Control organisms were treated identically. After irradiating, one ml. was removed from each plastic container, added to Warburg flasks as previously described and

TABLE II

The effect of various amino acids, glutathione and pyruvate on the respiration of control Tetrahymena and Tetrahymena exposed to 300,000 r. See text for concentration of supplement

Experiment No.	Supplement	Control QO_2	Irradiated QO_2	Inhibition (per cent)
1	——	27.8	17.6	36
	L-cysteine	34.4	28.3	18
	L-methionine	29.4	19.4	34
2	——	30.0	22.4	25
	L-cysteine	37.0	30.0	19
	Glycine	28.9		
3	——	24.4	12.8	48
	L-cysteine	35.4	28.8	19
	DL-homocysteine	24.6	20.8	15
4	——	24.3	13.1	46
	L-cysteine	34.6	31.8	8
5	——	16.5	13.6	18
	Glutathione	19.0		
	Glycine	16.5		
6	——	23.8	16.7	30
	DL-serine	26.5	20.1	24
	Sodium pyruvate	31.0	28.6	8

the oxygen consumption followed for one hour. The results of these studies are listed in Table II. L-cysteine (8.3×10^{-3} *M*) afforded 54 per cent protection (average of four experiments). In experiment 3 it can be seen that DL-homocysteine was equally effective at the same concentration. DL-serine (16.6×10^{-3} *M*) was only slightly effective while L-methionine (6.7×10^{-3} *M*) gave no pro-

TABLE III

RNase and DNase activity of homogenates prepared from normal and irradiated cells. Figures give specific activity

Dose (r)	RNase		Change (per cent)	DNase		Change (per cent)
	Control	Irradiated		Control	Irradiated	
300,000	0.162	0.147	−10	0.018	0.016	−11
....	0.204	0.204	0	0.018	0.018	0
....	0.099	0.104	+ 5	0.011	0.011	0
500,000	0.172	0.172	0	0.017	0.014	−18
RNase and DNase activity of irradiated homogenates						
500,000	0.127	0.054	−57	0.015	0.007	−52
....	0.144	0.082	−43			
....	0.151	0.078	−48			

tection. Sodium pyruvate (1.0×10^{-2} *M*), which is rapidly oxidized by Tetrahymena (Seaman, 1949), gave marked protection also.

It is to be noted that L-cysteine (final concentration 3.0×10^{-3} *M*) stimulated the respiration of normal cells by approximately 35 per cent, while glutathione in equal concentration increased the oxygen consumption by 15 per cent. It is possible that the tripeptide was hydrolyzed, liberating cysteine which was then oxidized. L-methionine (2.2×10^{-3} *M*) and DL-serine (6.0×10^{-3} *M*) augmented

TABLE IV

The effect of L-cysteine on RNase activity of homogenate irradiated at 500,000 r

Experimental conditions	Specific activity	Inhibition (per cent)
Control	0.144	——
X-irradiated	0.082	44
Control + L-cysteine	0.156	0
X-irradiated + L-cysteine	0.156	0

the respiration of normal cells slightly, while glycine (1.0×10^{-3} *M*) and DL-homocysteine (3.0×10^{-3} *M*) had no effect. No auto-oxidation of either L-cysteine or glutathione was observed under the test conditions.

Nuclease determinations. It is apparent from the data presented in Table III that the activities of both RNase and DNase were little changed in homogenates obtained from whole cells immediately after irradiation at 300,000 and 500,000 r. However, when homogenates were prepared from normal organisms and then

TABLE V

The effect of p-chloromercuribenzoate on RNase activity of homogenates irradiated at 500,000 r
See text for description of experiment

Experimental conditions	Specific activity	Inhibition (per cent)
1. Control	0.151	—
2. X-irradiated	0.078	48
3. Control + p-chloromercuribenzoate	0.118	25
4. X-irradiated + p-chloromercuribenzoate	0.036	76
5. X-irradiated + p-chloromercuribenzoate + L-cysteine	0.078	48

irradiated at 500,000 r, both enzymes were markedly inhibited. Irradiation of normal homogenates at 500,000 r in the presence of 3.6×10^{-2} *M* L-cysteine completely protected the RNase (Table IV).

In the light of the importance placed on the relationship between SH-dependent enzymes and *in vitro* inhibition due to ionizing radiations (Barron *et al.*, 1948–49), it seemed desirable to determine whether the RNase obtained from *T. geleii* W was an SH-dependent enzyme. The homogenate was divided into five 2-ml. por-

tions. Before irradiating with 500,000 r, sample 2 received 2 ml. of distilled water and samples 4 and 5, 2 ml. each of sodium p-chloromercuribenzoate (final concentration 2.6×10^{-3} M). Samples 1 and 3 were controls without and with p-chloromercuribenzoate. All samples were adjusted to the same pH. Three ml. of each of these solutions were used in the RNase assay which was standard except that 13.4 mg. of L-cysteine were added to the system prepared from sample 5. From the data presented in Table V, it can be seen that the addition of p-chloromercuribenzoate to unirradiated homogenate inhibited the enzyme by 25 per cent. RNase inhibition due to the presence of the SH-binding agent and the effect of x-irradiation was additive, the sum amounting to 76 per cent. The addition of L-cysteine to a mixture of homogenate and p-chloromercuribenzoate immediately after irradiation reversed the effect of the SH-reactant completely but did not alter the inhibition due to radiation.

Discussion

The question of whether the decreased oxygen consumption exhibited by irradiated Tetrahymena is a reflection of the inhibition of oxidative enzymes, mortality, or both must await further investigation. At 500,000 r, mortality is probably a significant factor. Elliott and Slater (1951) have reported that Tetrahymena were killed at 550,000 r. We have observed repeatedly that immediately after irradiating a solution containing 2×10^6 cells per ml. with 500,000 r, only an occasional organism exhibited motility; within 24 hours more of the cells regained the ability to move. However, in the light of only a 40 per cent reduction in respiration at 500,000 r, these observations may imply that many of the non-motile cells were alive and their respiratory processes inhibited. The protection against respiratory inhibition which was afforded the organisms by irradiating them in the presence of cysteine and homocysteine is another illustration of the ability of thiol compounds to antagonize the effects of ionizing radiations. The protection of RNase against x-ray inactivation by the addition of cysteine to homogenates is probably a similar phenomenon. This finding is in good agreement with the report of Holmes (1950) who showed that x-ray inactivation of crystalline pancreatic RNase in dilute aqueous solution could be prevented by glutathione. The protective action exerted by sodium pyruvate against respiratory inhibition is interesting in view of the finding that pyruvate will protect bacterial cells against the lethal action of x-rays and H_2O_2 (Thompson, Mefferd and Wyss, 1951; Hollaender, Stapleton and Burnett, 1951).

The significant difference observed between the *in vitro* and *in vivo* action of x-radiation on the activities of RNase and DNase is also of considerable interest. Since the intact Tetrahymena occupy roughly 5 per cent of the volume of the irradiated solutions, it may be that 95 per cent of the radio-decomposition products formed in the extracellular water are excluded by the cell membranes. Also, the oxygen tension within the cells is presumably lower than in the external water; thus fewer free radicals would be produced *in vivo*. Rupture of the cell membranes by homogenization would expose the cellular enzymes to the action of the entire quantity of injurious products formed.

From the inhibition of RNase by p-chloromercuribenzoate, it would seem that in Tetrahymena this enzyme is SH-dependent. Crystalline pancreatic RNase (calf) has been shown to be inhibited by several SH-compounds (Zittle, 1946).

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Summary

1. The respiration of x-irradiated *Tetrahymena geleii* W decreased 30 per cent at 300,000 r and 40 per cent at 500,000 r. The addition of cysteine to the organisms prior to irradiation at 300,000 r afforded approximately 50 per cent protection as measured by oxygen consumption. Homocysteine and pyruvate also gave marked protection while methionine had no effect. Cysteine and glutathione stimulated the respiration of normal cells appreciably, but methionine, serine, glycine and homocysteine had little or no effect.

2. Ribonuclease and desoxyribonuclease activities were not changed significantly in homogenates obtained from whole cells which were irradiated at 300,000 and 500,000 r. However, both enzymes were inhibited by about 50 per cent in homogenates which were irradiated at 500,000 r. For RNase, this inhibition was completely reversed by irradiating in the presence of cysteine. Inhibition of RNase of normal Tetrahymena homogenate by p-chloromercuribenzoate was reversed by cysteine.

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